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Sputum Trace Metals Are Biomarkers of Inflammatory and Suppurative Lung Disease

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Background: Induced sputum cytology and protein biomarkers can be used to assess airways inflammation. Increases in sputum iron have been described in inflammatory lung disease. We hypothesized that other sputum metals may be affected by airways inflammation and investigated their potential value as biomarkers.

Methods: Sputum was obtained from 20 healthy control subjects and from patients with inflammatory pulmonary diseases (23 with cystic fibrosis [CF], 16 with bronchiectasis, 17 with asthma, and 23 with COPD), and iron, zinc, manganese, and copper were measured. Fourteen patients with CF were also studied through an exacerbation cycle.

Results: Sputum zinc and iron were elevated in CF and non-CF bronchiectasis vs controls ($P < .001$, zinc; $P < .01$ iron). Manganese was elevated in asthma ($P < .01$) and bronchiectasis ($P < .05$) vs controls. Copper was elevated in CF vs controls ($P < .05$). Zinc decreased ($P < .01$) following treatment of CF exacerbation. In subjects with CF zinc levels correlated with other biomarkers.

Conclusions: These results suggest a relationship of high concentrations of total zinc and iron with airways inflammation in CF and non-CF bronchiectasis, with longitudinal changes being observed in CF. Further work is required to elucidate potential inflammatory mechanisms related to these observations.

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Abbreviations: CF = cystic fibrosis; IL = interleukin; MPO = myeloperoxidase; PBS = phosphate-buffered saline; SOD = superoxide dismutase

The presence of a proinflammatory milieu in the airway is important for the development of a range of inflammatory lung diseases. Adequate assessment

of inflammatory cells, cytokines, chemokines, and antiinflammatory molecules is essential for understanding, monitoring, and treating these disorders. Induced sputum provides a noninvasive means of investigating airways inflammation.¹ Induced sputum has a fluid phase containing a heterogeneous mix of proteins and nonorganic substances. A number of cytokines, including interleukin (IL)-8, have been measured,² albeit with significant interstudy variation in reported levels.³ The nonorganic component, however, has been largely neglected.

Previous work performed by this group has demonstrated high levels of the neutrophil protein calprotectin in the sputum⁴ and lavage fluid⁵ of patients with CF and non-CF bronchiectasis. Calprotectin possesses antimicrobial properties mediated by the chelation of zinc.^{6,7} It has been demonstrated recently that calprotectin is recruited to staphylococcal abscesses in a neutrophil-dependent manner and inhibits *Staphylococcus*

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aureus growth through the chelation of zinc and manganese.⁸

Increased levels of total iron and iron-binding proteins have been reported in the sputum of patients with cystic fibrosis (CF) and COPD.⁹⁻¹² Scavenging of free iron is an important component of antimicrobial defense mechanisms against organisms such as *Pseudomonas aeruginosa*,¹³ an important pathogen in chronic lung disease. Although iron is clearly an important inorganic substance in the airway, other metals, such as zinc, may also be important; for example, increased zinc levels alter the sensitivity of *Pseudomonas*, an organism particularly relevant to CF lung disease, to antibiotics.¹⁴ Therefore the measurement of trace elements in sputum may allow mechanistic insight into diseases such as CF. Furthermore, trace elements, likely to be less susceptible to protease activity, may allow more reliable measurement of inflammation in the airway. Thus we feel that measurement of trace elements may allow a more robust sputum measurement of airways disease as well as giving further insight into lung inflammation in diseases such as CF.

Based on our previous findings of increased levels of calprotectin, a known chelator of zinc, in the airway and previous observations related to iron in the CF airway we hypothesized that trace metal concentrations, in particular zinc and iron, would be elevated in CF and non-CF bronchiectasis compared with control populations. We also hypothesized that levels of trace element would change following disease intervention in CF. Total concentrations of zinc, iron, copper, and manganese were measured in subjects with CF, bronchiectasis, COPD, and asthma, and in normal controls. Furthermore, concentrations of trace elements were compared with previously described markers of inflammation in CF, including calprotectin.

MATERIALS AND METHODS

Subjects

Induced sputum was obtained from patients attending the Respiratory Unit at the Western General Hospital, Edinburgh. Approval was obtained from the local regional ethics committee. Twenty-three patients with CF, 16 with non-CF bronchiectasis, 17 with asthma, 23 with COPD, and 20 healthy controls were studied. In order to monitor the effects of a changing state of inflammation, sputum samples were obtained from 14 additional patients with CF during CF exacerbation. Exacerbation samples were taken within 24 hours of starting antibiotic therapy and at the end of treatment.

Reagents Used

All reagents were purchased from Sigma (Sigma; Gillingham, UK) unless otherwise stated. Ultrapure, high-performance liquid chromatography-grade water was used for all experiments and preparations. Aristar nitric acid was used in the trace element assay.

Sputum Induction

Sputum induction was performed by a standard method as previously described.¹⁵⁻¹⁷ In brief, patients inhaled nebulized hypertonic saline at concentrations of 3%, 4%, and 5% and were asked to expectorate after each inhalation until a sample was obtained. Patients with CF underwent induction unless freely expectorating in keeping with previous CF sputum studies.^{18,19} Nine of 23 subjects with CF spontaneously produced sputum without induction.

Sputum Processing

Sputum was processed within 2 hours of collection as described previously.¹⁵ In brief, sputum plugs were harvested and processed with 4 × weight/volume of 0.1% dithiothreitol in phosphate-buffered saline (PBS), after which 4 × weight/volume of PBS was added. Samples were filtered through 48-μm mesh and centrifuged at 1,200 rpm to remove the cells. Supernatant was stored at -80°C until further analysis without protease inhibitor. The cell pellet was resuspended in PBS and used for cytospin preparation. Cytospins were stained with May-Grunwald-Giemsa for differential cell counting. All cell counts were expressed as percentage of the population counted. Total cell count was not performed prior to cytospin. All samples used in this study contained < 40% squamous cells.

Trace Element Assay

Trace element assay was performed in the Scottish National Trace Element Reference Laboratory, Glasgow, Scotland. A four-point calibration was used (0, 100, 500, 1,000 μg/L copper, iron, zinc, manganese in 1% nitric acid). Sputum samples were centrifuged at 800 g for 5 min and 200 μL of sample was then diluted with 2 mL internal standard solution (100 μL yttrium in 1% nitric acid) and mixed by inversion. Internal accuracy was assessed by use of two aqueous certified reference materials, TMDA62 and TMDA64 (Prochem; Chessington, England). Trace element levels were measured by inductively coupled plasma optical emission spectrometry using a VISTA AX (Varian; Oxford, England).

Measurement of Sputum IL-8, Myeloperoxidase, and Calprotectin

To compare the results of metals in sputum with biochemical markers of inflammation, comparisons with IL-8, myeloperoxidase (MPO), and calprotectin were made in the CF group. Immunoassays used commercially available sandwich enzyme-linked immunosorbent assay kits, following the manufacturer's instructions. The kits used were IL-8 (Biosource Europe SA; Nivelles, Belgium), MPO (Assay Designs Inc; Ann Arbor, MI), and Calprotectin (Bühlmann Laboratories, AG; Schönenbuch, Switzerland).

Data Analysis

Data analysis was carried out on GraphPad Prism software (GraphPad; La Jolla, CA) for Windows. Cross-sectional data were nonnormally distributed and were analyzed by Kruskal-Wallis analysis of variance and Dunn multiple comparison test. $P < .05$ was considered significant. For longitudinal analysis of zinc and iron data a paired Student *t* test was performed. For correlation, data were subjected to Spearman rank analysis.

RESULTS

Subject Demographics

The demographic profiles and sputum cytology of each group are shown in Table 1. The CF group was

Table 1—Demographics and Sputum Cytology for Subjects in Cross-Sectional and Longitudinal Studies

Disease	No.	Male, No.	Age, y	FEV ₁ % Predicted	Neutrophil, %	Eosinophil, %
Controls	20	6	36.9 (2.5)	105.8 (2.6)	55 (4.3)	2.9 (0.5)
Asthma	17	5	51.9 (3.3)	80 (5.6)	60 (6.1)	16.2 (5.2)
COPD	23	16	66.0 (1.4)	55.0 (4.0)	81 (2.6)	4.4 (1.6)
CF	23	14	26.3 (2.0)	59.1 (4.4)	92 (2.0)	2.4 (0.5)
Bronchiectasis	16	1	62.3 (2.1)	70.4 (8.5)	84 (4.0)	2.1 (1.3)
CF exacerbation	14	8	25.6 (2.4)	41.0 (4.4)	98.3 (0.6)	n/d
CF recovery	14	8	25.6 (2.4)	46.3 (4.5)	96.4 (0.9)	n/d

Data are displayed as mean (SEM). CF = cystic fibrosis; n/d = not done.

younger than the control and other disease groups ($P < .01$). Of the COPD group 10 were current smokers, nine ex-smokers, and four gave no information on current smoking status. Of the patients with CF, 14 were colonized with *P aeruginosa*, the other patients being colonized by a variety of organisms, including *Stenotrophomonas maltophilia* and *Burkholderia cenocepacia* species.

Assay Reproducibility

Pooled samples of five subjects in each group of control, CF, and non-CF bronchiectasis were assayed in two separate runs for zinc levels with coefficients of variance of 14.7%, 2.7%, and 5.4%, respectively, giving an average coefficient of variation of 7.6%.

Sputum Trace Element Levels in Cross-Sectional Data

The absolute concentrations of zinc, iron, and manganese are displayed in Table 2 in $\mu\text{g/L}$. Sputum zinc concentration was at least fourfold higher in CF and non-CF bronchiectasis than controls ($P < .001$). Concentrations in CF and non-CF bronchiectasis were also higher than in asthma and COPD ($P < .05$).

Sputum iron was at least twofold higher in CF and non-CF bronchiectasis than controls ($P < .01$) and COPD ($P < .05$). Levels were higher in the COPD and asthma groups vs controls but did not reach statistical significance. Current smoking status had no

significant effect on the zinc or iron levels in the COPD group (Fig 1).

Sputum manganese differentiated non-CF bronchiectasis but not CF from control ($P < .05$). Sputum manganese also differentiated subjects with asthma from control subjects ($P < .01$). Sputum copper was higher in all disease groups vs control but only reached statistical significance for CF ($P < .01$).

In some subjects trace element levels were below the limit of detection of the assay, in particular for manganese. Interestingly, zinc and iron were detectable in all subjects with CF. Please refer to Table 2 for further information.

Correlation of Sputum Zinc and Iron Levels With Lung Function, Sputum Cytology, and Sputum Biomarkers in Patients With CF

As the most statistically significant changes were seen in the CF group, further comparisons were made with clinical data and other inflammatory markers for this group. Sputum zinc and iron levels were correlated in patients with CF (Spearman $r = 0.75$, $P < .05$, data not shown). There was a negative correlation of zinc and FEV₁% predicted in the CF group (Spearman $r = -0.469$, $P < .05$, Fig 2A). Sputum iron was also negatively correlated with FEV₁% (Spearman $r = -0.43$, $P < .05$, data not shown). Sputum neutrophil % and zinc levels were correlated (Spearman $r = 0.67$, $P < .05$, data not shown). Zinc

Table 2—Levels of Metal Elements ($\mu\text{g/L}$) of Processed Sputum Supernatant for Zinc, Iron, Manganese, and Copper

Disease	Zinc	Iron	Manganese	Copper
Control	15.35 (10.4-25.6)	13.5 (8.6-21.5)	0 (0-0.25)	8.6 (3-16.4)
Asthma	12.7 (7.2-41.4)	30.0 (6.9-35.3)	0.8 (0.2-1.7) ^a	15.2 (8.6-29.5)
COPD	25.4 (9.8-50.7)	21.3 (3.1-35.6)	0 (0-0.7)	15.2 (12.2-22)
CF	135.3 (54.2-209.6) ^b	56.9 (24.3-115.3) ^a	0.3 (0.1-0.8)	19.5 (14.5-30.1) ^c
Bronchiectasis	111.3 (46.1-150.7) ^b	54.2 (22.7-91.6) ^a	0.6 (0.2-1.3) ^c	15.7 (10.9-33.3)

Data are displayed as: median (interquartile range). P values are displayed for disease group vs control; for P values between groups please consult text. Zinc was below the limit of detection of the assay in four control and one COPD. Iron was below the limit of detection in four control, one asthma, and one COPD. Manganese was below the limit of detection in 14 control, three asthma, 13 COPD, six CF, and one bronchiectasis. See Table 1 for expansion of abbreviation.

^a $P < .01$ vs control.

^b $P < .001$ vs control.

^c $P < .05$ vs control.

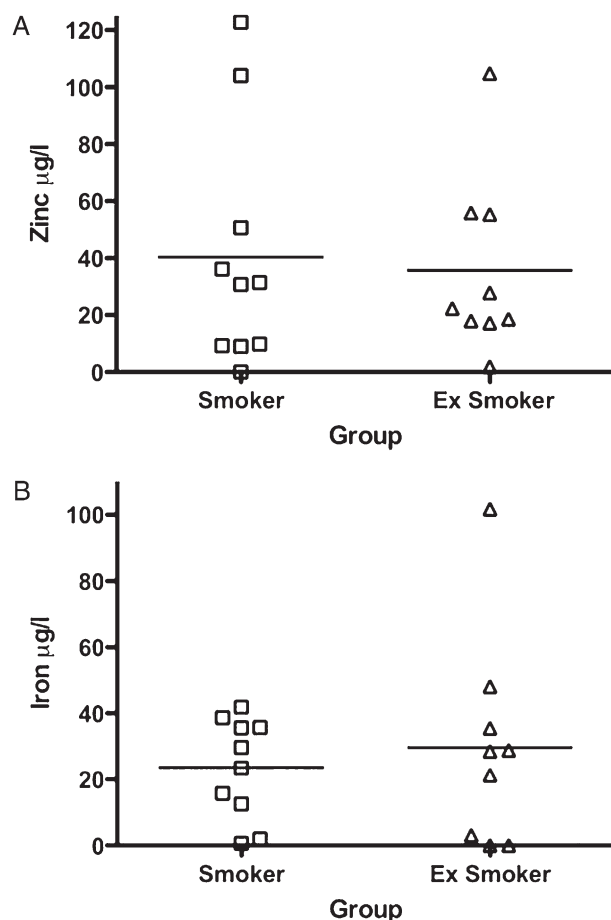


FIGURE 1. Smoking status and sputum trace element level in COPD. Data for zinc (A). Data for iron (B).

levels were unrelated to the underlying colonizing organism (data not shown).

Sputum zinc and calprotectin levels correlated with high statistical significance (Spearman $r = 0.86$, $P < .001$, Fig 2B). Sputum zinc significantly correlated with MPO and IL-8 (Spearman $r = 0.81$, $P < .001$, Spearman $r = 0.67$, $P < .001$, respectively, Figures 2C and 2D). Sputum iron demonstrated similar but less significant correlations to calprotectin, MPO, and IL-8 (data not shown).

Serial Measurement of Metals in CF Exacerbation

Sputum zinc levels decreased significantly following antibiotic therapy for an exacerbation ($P < .01$; Fig 3). There were no significant serial changes in iron, manganese or copper.

DISCUSSION

Total elemental zinc and iron concentrations are elevated in sputum from subjects with CF and non-CF bronchiectasis compared with healthy control

subjects. There is a small degree of overlap between the CF and control groups, but this may be explained by the finding that patients with CF in this small subgroup had better lung function and thus less severe lung disease (data not shown). Sputum zinc levels were also significantly higher in CF and non-CF bronchiectasis compared with COPD. Sputum zinc levels decrease significantly over the course of a CF exacerbation. Zinc is strongly correlated with calprotectin in CF sputum as well as with other inflammatory markers, such as IL-8 and MPO.

Protein biomarkers, such as cytokines, have been used in previous studies to assess levels of airways inflammation.²⁰⁻²² However, protease activity in expectorated sputum may affect the robustness of cytokine assays.²³ The potential association we have shown between trace element levels and inflammation in sputum samples combined with their likely resistance to degradation commends them as potentially robust markers of lung pathophysiology.

Serum zinc has previously been suggested as a marker of lung disease,^{24,25} but we believe this to be the first study to describe an association between sputum zinc and inflammation in individuals with CF lung disease and non-CF bronchiectasis. Furthermore, we demonstrate its potential use as a serial marker during treatment of an exacerbation, although this must be interpreted with caution as in two subjects zinc levels actually increased, whereas in four others there was only a modest decrease of levels. Nevertheless, the majority of subjects demonstrated a decrease with treatment and as such further investigation is clearly merited.

We thus suggest that sputum zinc may be used as a biomarker in suppurative diseases, such as CF. We do realize, however, that as we have used a relatively small sample size, albeit similar to those use in previous biomarker studies, these data represent a novel observation and further validation in larger studies is required. Furthermore, a parallel measure of zinc in serum may have added further insight into the functional significance of these findings, as would knowledge of individual subject dietary zinc intake.

Zinc homeostasis may play an important role in modulating the immune response to inflammation, with high concentrations of zinc inducing peripheral blood monocyte apoptosis²⁶ and promoting cytokine production.²⁷ Conversely, low concentrations of zinc may suppress monocyte function and decrease neutrophil phagocytosis.²⁸ Zinc may also interact with the airway epithelium.^{29,30} For example, zinc deprivation of bronchial epithelial cells in culture induces apoptosis,^{31,32} and zinc deficiency in a murine model of asthma induces epithelial cell apoptosis and airways inflammation.³³ We have measured total zinc content of sputum (ie, bound and unbound), whereas

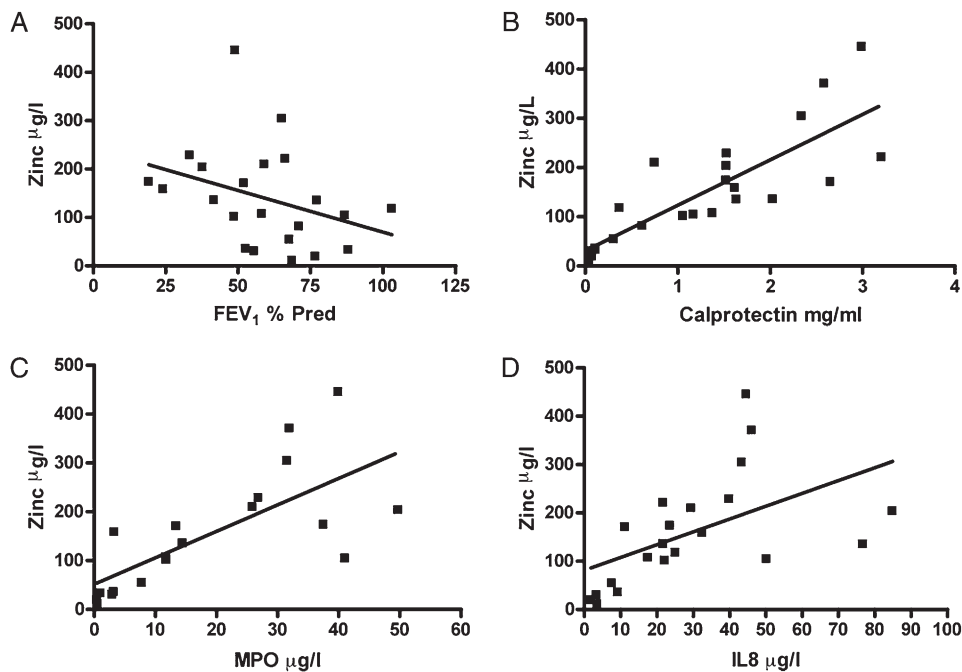


FIGURE 2. Correlation of sputum zinc with biomarkers of lung inflammation and physiology. Correlation between FEV₁% predicted and sputum zinc (Spearman $r = -0.47$, $P < .05$) (A). Correlation between sputum calprotectin and zinc. (Spearman $r = 0.86$, $P < .001$) (B). Correlation between sputum MPO and zinc (Spearman $r = 0.81$, $P < .001$) (C). Correlation between sputum IL-8 and zinc (Spearman $r = 0.67$, $P < .001$) (D). IL = interleukin; MPO = myeloperoxidase.

the amount of freely available unbound zinc might be equally important. The excess of zinc in sputum is, however, a possible explanation for the low serum zinc levels in subjects with CF compared with healthy controls.³⁴

Increases in sputum iron have been described in CF⁹⁻¹² and exceed the levels observed in COPD.¹² Iron is a

prerequisite for microbial growth, with increased levels in CF sputum possibly contributing to the proliferation of bacteria such as *P. aeruginosa*.¹¹ The source of this iron is unclear, with leak from the circulation being suggested, although further work is required to investigate this. Cigarette smoke has also been suggested as a potential source of airways iron,³⁵ but we have demonstrated no difference in sputum iron between current smokers and ex-smokers with COPD in this study.

Associations between the levels of sputum manganese and copper with disease type are less obvious. Manganese was only significantly elevated in the asthma and non-CF bronchiectasis groups and copper only in CF. Both metals (like zinc) are cofactors for superoxide dismutases (SODs), which have leading roles in alleviating oxidative stress in the lung.³⁶ Furthermore, the demonstration that manganese differentiated subjects with asthma from control subjects but not subjects with CF from control subjects suggests a possible role for this element in nonsuppurative lung disease. SODs are downregulated in asthmatic airways,³⁶ suggesting sputum copper and manganese are not merely tracking levels of these mediators. A measurement of SOD level or activity would help to elucidate this complicated relationship but was not performed in this study and thus forms the basis of future work by this group.

Sputum zinc levels correlated with biomarkers of airways inflammation, namely calprotectin, MPO,

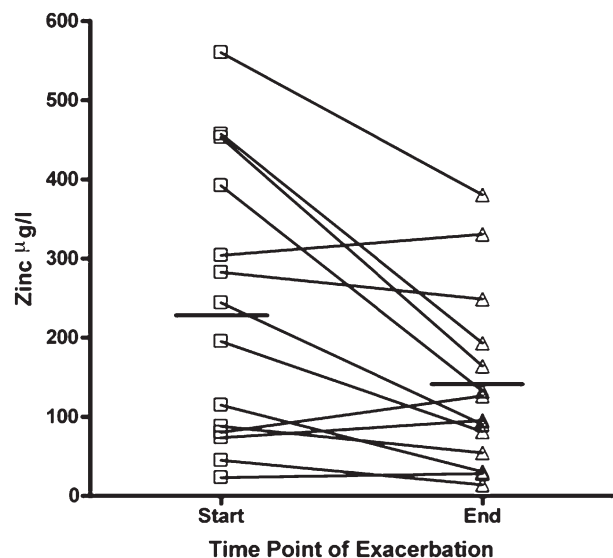


FIGURE 3. Sputum zinc levels in CF exacerbation ($n = 14$). Sputum zinc decreases over the course of an exacerbation with antibiotic treatment ($P < .01$). Horizontal lines represent means. CF = cystic fibrosis.

and IL-8. Sputum zinc adds to the growing number of potential biomarkers in sputum and may be seen to compliment these as well as offering new insight into pulmonary inflammation. Calprotectin is a highly abundant neutrophil protein found in the CF airway^{4,5} with antiinflammatory and proinflammatory functions, and the ability to chelate zinc and other cations.^{7,8,37,38} Calprotectin promotes apoptosis in cell lines via the exclusion of zinc,^{39,40} and zinc and calprotectin have been demonstrated to colocalize in staphylococcal abscess in a murine model.⁸ As such, we would suggest that the interaction of zinc and calprotectin in the CF airway is of mechanistic importance, particularly when we consider that *S aureus* is a major pathogen in early disease.

Elevated levels of calprotectin and zinc may reflect passive release of the neutrophil contents in view of the large number of necrotic neutrophils in the CF airway,⁴¹ or may represent active secretion as is observed with lactoferrin release from neutrophils.⁴² A passive release of zinc, on cell necrosis, would be supported by the correlation of zinc with both neutrophil percentage in samples and calprotectin, a cytoplasmic protein in neutrophils. A more controlled active release of zinc may be suggested, however, by the correlation of zinc with MPO, a neutrophil granule protein released from activated neutrophils. Nevertheless, the higher correlation is of zinc, and calprotectin might simply reflect an overall abundance of neutrophils, which are rich in both of these substances,^{43,44} as neutrophils contain 5 to 10 ng zinc per 10⁶ cells.⁴³ It is also important to consider that the zinc we have measured in the airway could also be complexed to calprotectin (or other proteins) or could be due to leakage from the pulmonary circulation during inflammation.

In conclusion, we have demonstrated elevated levels of trace metals (zinc in particular) in the fluid phase of sputum from patients with CF and non-CF bronchiectasis compared with patients with asthma or COPD and healthy adult controls. We also demonstrate that the level of zinc in CF sputum decreases over the course of an infective exacerbation and the use of chemically stable markers in noninvasive assays to monitor the course and severity of lung diseases such as CF would clearly be advantageous. Of course a valid biomarker must be highly reproducible and repeatable and, as such, longitudinal studies to evaluate the robustness of sputum trace metal assays of inflammation are required.

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Author contributions: *Dr Gray:* contributed to the original concept and design of the study, manuscript writing and preparation, and collecting, processing, and analyzing samples.

Dr Duncan: contributed to overseeing trace element analysis and providing intellectual input to the manuscript.

Dr Noble: contributed to collecting, processing, and analyzing samples.

Ms Imrie: contributed to collecting, processing, and analyzing samples.

Dr O'Reilly: contributed to overseeing trace element analysis and providing intellectual input to the manuscript.

Dr Innes: contributed to providing senior mentorship and intellectual input and was involved in manuscript writing.

Dr Porteous: contributed to providing senior mentorship and intellectual input and was involved in manuscript writing.

Dr Greening: contributed to providing senior mentorship and intellectual input and was involved in manuscript writing.

Dr Boyd: contributed to the original concept and design of the study and manuscript writing and preparation.

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